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14. ABSTRACT
The role of complement in cancer metastasis has not yet been recognized. In addition, a role of adaptive immunity in distant from primary tumor sites in preventing metastasis is unclear. Utilizing a model of breast cancer, we found that the complement anaphylatoxin C5a receptor (C5aR) facilitated lung and liver metastasis by suppressing effector $CD8^+$ and $CD4^+$ T cell responses. Mechanisms of this suppression involved recruitment of immature myeloid cells to distant sites and regulation of TGF-β and IL-10 production in these cells. TGF- β and IL-10 favored generation of Regulatory T (T_{reg}) cells and Th2 predominant responses that rendered $CD8^+$ T cells dysfunctional. Importantly, pharmacological blockade of C5aR or its genetic ablation in C5aR-deficient mice reduced metastases. Depletion of $CD8^+$ T cells abolished this beneficial effect suggesting that $CD8^+$ T cells are responsible for C5aR inhibition-dependent reduction in metastasis. In contrast to previous findings, C5aR-signaling appeared to promote T_{reg} cell generation and suppress T cell responses in metastases-targeted organs. These findings indicate that immunomodulatory functions of C5aR are highly context dependent. Furthermore, these data open a new avenue for developing complement-based immunotherapies to prevent or reduce cancer metastasis

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Introduction

Studies proposed in this application have been designed to address whether blocking of the complement anaphylatoxin C5a receptor (C5aR): improves the effectiveness of anti-Her2/neu immunization in inducing the regression of primary breast tumors in a transgenic model of breast cancer (Aim 1), reduces the extent of metastatic spread of breast carcinoma and improves the effectiveness of anti-Her2/neu immunization in limiting the growth of breast cancer metastases in a model of spontaneously metastasizing breast cancer (Aim 2). We also dissect mechanisms by which C5aR blockade improves efficacy of anti-Her2/neu immunization in curing advanced breast carcinoma and its metastases (Aim 3). This research involves transgenic and syngeneic models of breast cancer. Tumor bearing mice have been subjected to treatment with various combinations of C5aR inhibitor and Her2/neu-targeting vaccine. The impact of these treatments on tumor growth has been monitored and various features of anti-tumor immune responses and immunosuppression mechanisms have been evaluated.

Body:

The specific aims of this application are:
Aim 1 Determine whether blocking C5aR improves the effectiveness of anti-Her2/neu immunization in inducing the regression of primary breast tumors. We anticipate achieving this aim within the first year of the project (**months 1-12**).
Aim 2 Determine whether blocking C5aR: (i) reduces the extent of metastatic spread of breast carcinoma and (ii) improves the effectiveness of anti-Her2/neu immunization in limiting the growth of breast cancer metastases. We anticipate achieving this aim in the second year of the project (**months 12-24**).
Aim 3 Dissect mechanisms by which C5aR blockade affects the results of anti-Her2/neu immunization in inducing the regression of or limiting growth of breast carcinoma and its metastases. We anticipate conducting studies in this aim throughout the entire funding period (**months 1-24**).

2012-2013
For the previous funding period (3/2012-2/2013), we reported that blockade of the complement anaphylatoxin C5a receptor (C5aR) reduced tumor growth in syngeneic and Her2 transgenic mouse models of breast cancer. In both models the therapeutic efficacy of C5aR inhibitor was comparable to the efficacy of *Listeria monocytogenes*-delivered Her2 vaccine (Lm-LLO-Her2). Importantly, C5aR inhibition synergized with Lm-LLO-Her2 in limiting tumor growth. These therapeutic effects were associated with the enhanced recruitment of tumor-specific $CD8^+$ T cells to tumors. Notably, C5aR inhibition alone contributed to this recruitment and induced tumor-specific T cell responses at the periphery. The induction of the robust anti-tumor T cell responses by various treatments resulted likely from the attenuation of tumor mediated immunosuppression, since we observed that Lm-LLO-Her2, C5aR inhibition and the combination of Lm-LLO-Her2 with C5aR inhibition reduced infiltration of tumors by myeloid-derived suppress cells (MDSCs). The C5aR blockade impacted MDSC infiltration of tumors more than Lm-LLO-Her2. Overall, these data indicated that complement inhibition could become an efficient immunotherapy for breast cancer patients in a form of monotherapy or in the combination with other treatment modalities and addressed **Aim 1** and **3** of the original application.

2013-1014
In this funding period (3/2013-2/2014) we focused on **Aim 2** and A**im 3** of the original application. The description of results is preceded by the specific aims and tasks from the statement of work, included in the original application, to which these results pertain.

Aim 2 (TASK 5: months 12-18; TASK 6: months 12-18; TASK 7; months 12-18) and Aim 3

Preventing cancer metastasis is a significant goal of cancer therapy, as the majority of cancer deaths are attributed to this process [1]. However, progress in this area is limited, as a result of our poor understanding of its mechanism. Recently, emerging evidence has indicated that in addition to the mechanisms operating in neoplastic cells [2], alterations in host homeostasis, particularly in the immune system, contribute to metastasis [3]. These alterations occur in the primary tumor microenvironment [2], however, the role of host-derived cells and mediators at the distant sites has also been emphasized [4]. According to the concept of premetastatic niche malignant tumors prepare distant organs to receive metastases by altering host homeostasis in these organs prior to tumor cell arrival. Since these changes precede metastases, therapeutic targeting of premetastatic niche might prevent metastasis. The existence of premetastatic niche was proposed over a hundred years ago [3], but only recently a few components of this niche have been identified including myeloid-derived suppressor cells (MDSCs) [5, 6, 7, 8]. The primary tumor hypoxia inducible factors [9], serum amyloid A3 induced by S100A8 and A100A9 [10], and S1PR1-STAT3 signaling [11] have been suggested to be involved in recruiting these cells from the bone marrow to premetastatic organs. However, mechanisms governing recruitment of various cells to premetastatic organs and how these cells facilitate metastases still require clarification. It is conceivable that MDSCs, which suppress anti-tumor T cell responses in primary tumors and peripheral lymphoid organs [12], shield metastasizing tumor cells from immune attack in distant sites targeted by metastases [5]. However, in contrast to primary sites, the significance of T cell suppression in premetastatic niche remains unclear [13]. Since the complement anaphylatoxin C5a, a potent chemoattractant in inflammatory reactions [14], activates and attracts immunosuppressive cells to primary tumors [15], we hypothesize that C5a also contributes to immunosuppression facilitating metastases in distant sites.

(1) C5aR signaling facilitates lung and liver metastases

C5aR-signaling was found to promote tumor growth by modulating anti-tumor immunity in a syngeneic model of cervical cancer [15]. However, its role in metastatic spread of cancer has not been explored. Therefore, we tested whether C5aR contributes to metastasis. We found that C5aR deficiency reduced lung (Fig. 1A, B) and liver (Fig. 1C, D) metastatic burden without significantly affecting the growth of primary breast tumors (Fig. 1E) in a syngeneic model of

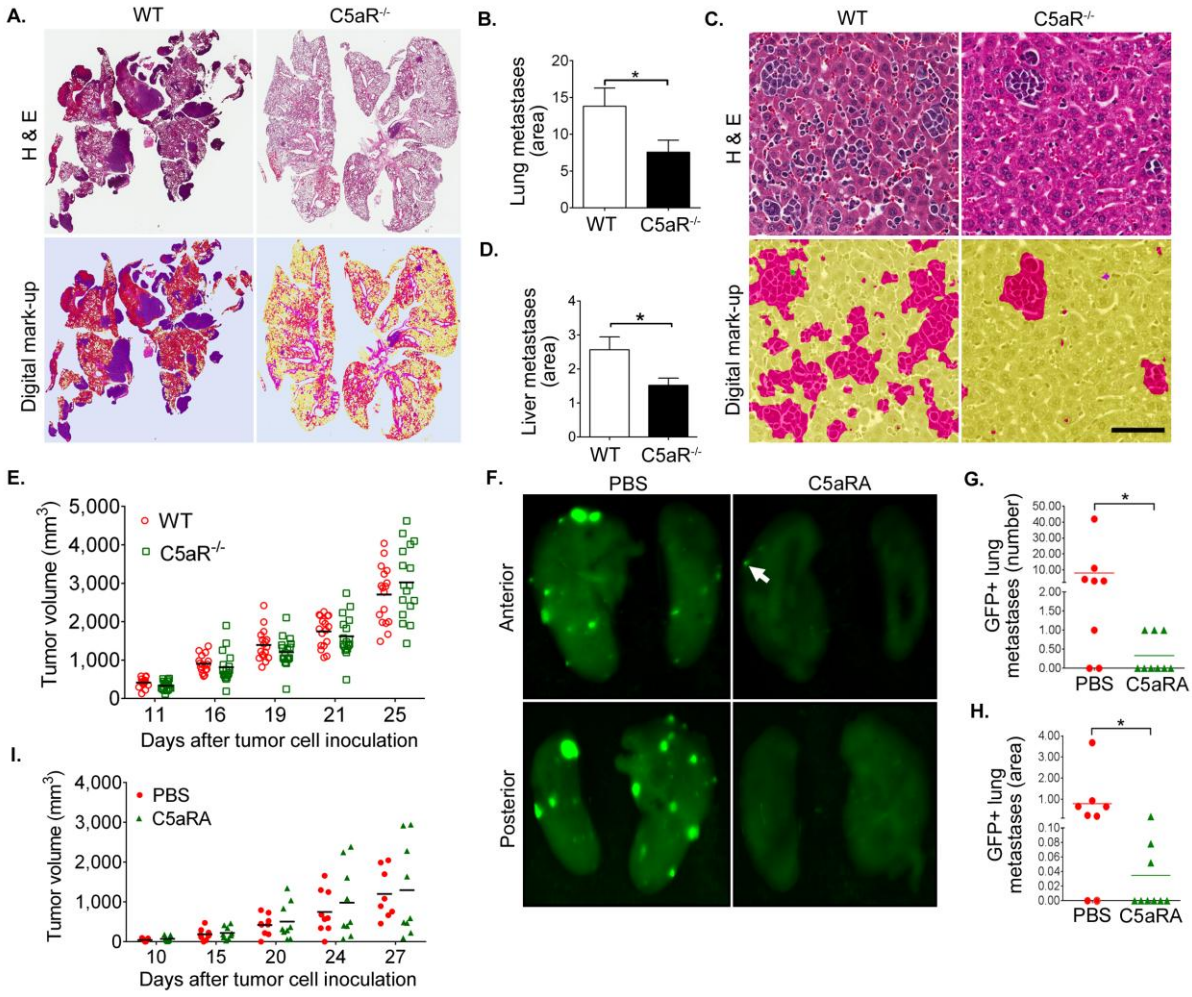

Figure 1. C5aR signaling promotes metastasis to the lungs and livers of breast tumor-bearing mice. Scans of hematoxylin and eosin (H&E) stained sections of the lungs (**A**) and livers (**C**) of wild-type (WT) and C5aR-deficient (C5aR$^{-/-}$) mice with breast tumors (upper panels) and their corresponding digital mark-up images (lower panel), metastases-purple, tissue with severe inflammatory changes- red, and remaining tissue-yellow and pink colorations. Quantification of lung, **P*=0.0421 (t-test) (**B**), and liver, **P*=0.0245 (t-test with Welch's correction) (**D**), metastases from A and C, respectively. Breast tumor volumes of WT and C5aR$^{-/-}$ mice at various time points after tumor cell injection (**E**). GFP^{+} metastases in the lungs of tumor-bearing mice treated with PBS or C5aR antagonist (C5aRA), arrow points to small metastatic tumor (**F**). Number of lung metastases, **P*=0.0225 (Mann Whitney test) (**G**), and area covered by these metastases **P*=0.0141 (Mann Whitney test) (**H**), in PBS and C5aRA-treated tumor-bearing mice. Breast tumor volumes of Balb/c mice treated with C5aRA or PBS at various time points after injection of tumor cells (**I**). Horizontal lines represent mean in scatter plots (**E, G-I**). Bars represent mean + s.e.m. Data are representative of two independent experiments with n_1=19, n_2=10 (**A-E**), or two independent experiments with $n_1 \geq 8$, n_2=10 (**F-I**). Scale bar-50 μm C.

breast cancer (4T1), which closely mimics the stage IV of human breast cancer [16]. Decreased metastatic burden together with the lack of an impact of C5aR-deficiency on primary tumor growth suggests that C5aR promotes metastasis through the mechanisms that are independent of those operating in primary tumors. In addition, since 4T1 tumor cells do not express C5aR (data not shown), C5aR-signaling in tumor cells does not directly govern metastasis to the distant organs. To support our data from genetically modified mice, we examined impact of pharmacological inhibition of C5aR on metastases in mice bearing GFP-expressing 4T1 breast tumors (4T1-GFP$^+$). Metastatic burden was markedly reduced in mice treated with C5aR antagonist (C5aRA) compared to placebo treated control mice (Fig. 1F-H). Importantly, 75% of the mice that received C5aRA remained metastases-free (Fig. 1G), while 25% of the mice developed fewer and smaller lung metastases than control mice (Fig. 1G, H). Noteworthy, albeit of a significant impact on metastasis, similar to the observations from the experiments with C5aR knockout mice, pharmacological inhibition of C5aR by C5aRA did not affect growth of the primary tumors in this study (Fig. 1I).

(2) C5aR inhibits the recruitment and function of CD4$^+$ and CD8$^+$ T cells in the lungs of breast tumor-bearing mice

Anti-tumor CD4$^+$ and CD8$^+$ T cells are considered as major effectors that limit tumor growth at the primary sites and our previous study linked C5aR to anti-tumor T cell responses [15]. However, the role of T cells at distant organs in preventing metastases has not been demonstrated. Therefore, we examined impact of C5aR blockade on both of these T cell populations. We found higher numbers of CD4$^+$ and CD8$^+$ T cells in the peripheral blood (Fig. 2A, E) and higher percentages of these cells in the lungs of breast tumor-bearing mice treated with C5aRA compared to control mice (Fig. 2B, F). A similar observation was made in C5aR$^{-/-}$ mice (data not shown). We hypothesize that these T cells, which were found to be more frequent in C5aR-deficient or C5aRA treated mice, would also be more efficient in the immunosurveillance of the distant organs, eventually contributing to reduction in metastatic burden. This hypothesis is supported by significantly higher percentages of IFN-γ producing CD4$^+$ and CD8$^+$ T cells observed in the lungs of C5aRA treated or C5aR-/- mice and when stimulated ex vivo with CD3/CD28 antibodies (Fig. 2C, G, D, H). Thus, we propose that the absence of C5aR-signaling encompasses Th1 and Tc1 predominant responses, which are likely to be involved in the clearance of circulating and/or seeding tumor cells in the lungs. This is further supported by significantly higher numbers of perforin-armed CD8$^+$ T cells infiltrating the lungs of breast tumor-bearing mice that received C5aRA (Fig. 2I, J), supporting contribution of these cells to protection of this organ against metastasizing tumor cells, since acquisition of perforin is a major effector function of CD8$^+$ cytotoxic T cells (CTL) and these cells posses tumoricidal activity [17]. To confirm that reduction in metastatic burden caused by C5aR inhibition depended on the protective role of CD8$^+$ T cells, we investigated the impact of C5aR inhibition on lung metastases in the mice that were depleted of CD8$^+$ T cells. C5aR blockade did not reduce lung metastases in these mice (Fig. 2K). On the contrary, in mice with an intact CD8$^+$ T cell population treated with control IgG, we observed the protective effect of C5aRA treatment, with a significant reduction in the lung metastatic burden compared to control mice

(Fig. 2K). This observation indicates that C5aR inhibits protective function of CD8⁺ T cells in the metastasis-targeted organs rendering them unable to control metastasis.

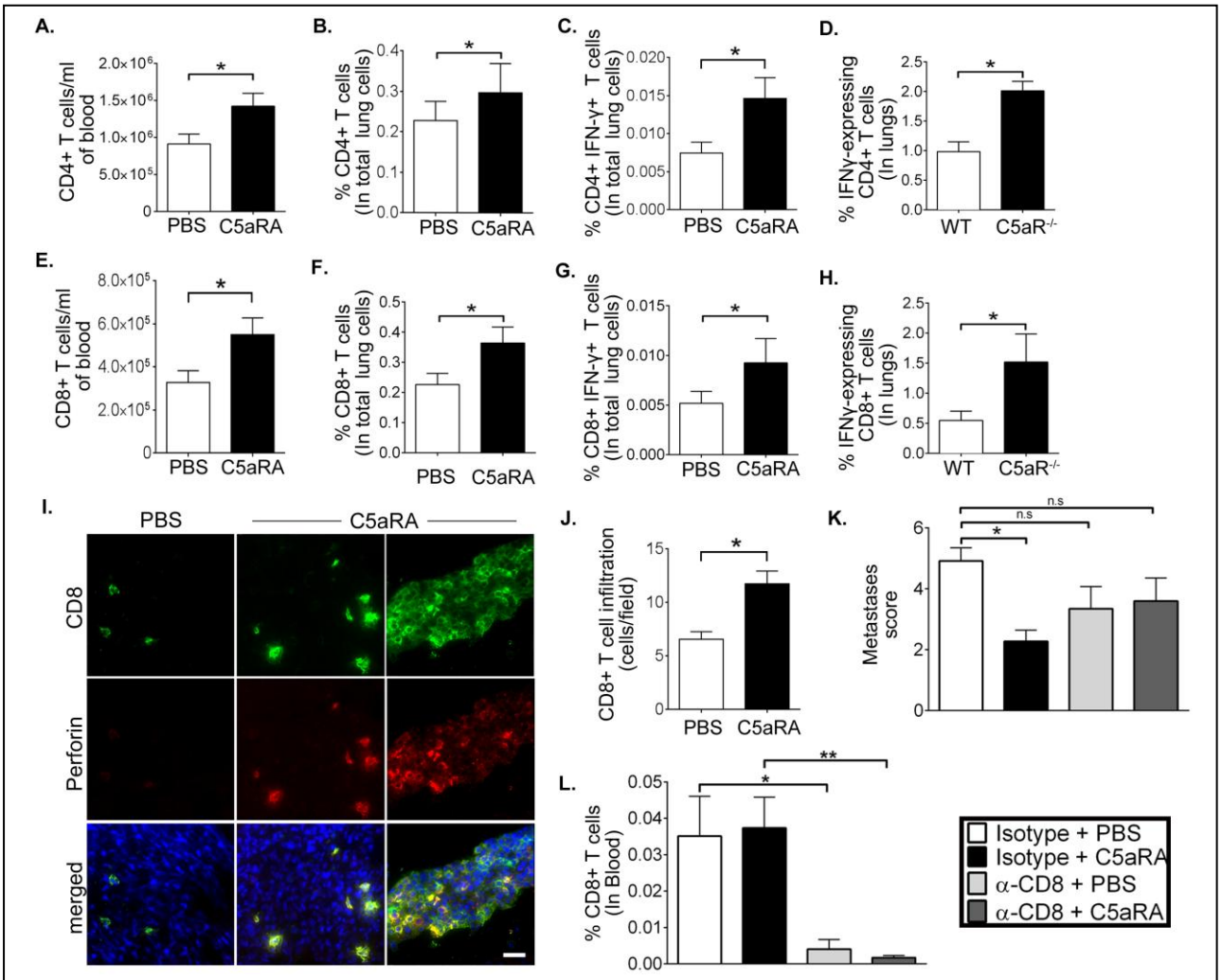

Figure 2. C5a receptor blockade enhances CD4⁺ and CD8⁺ T cell responses. CD4⁺ T cells from peripheral blood, **P*=0.0358 (t-test) (**A**), and lungs, **P*=0.2452, with 95% confidence interval (CI) -0.08 to 0.30 (t-test) (**B**), of breast tumor-bearing mice injected with PBS or C5aR antagonist (C5aRA). IFN-γ expressing CD4⁺ T-cells from lungs of breast tumor-bearing mice injected with PBS or C5aRA, **P*=0.0368 (t-test) (**C**), and WT and C5aR$^{-/-}$ mice, **P*=0.0004 (t-test) (**D**). CD8⁺ T cells from peripheral blood, **P*=0.0267 (t-test) (**E**), and lungs of breast tumor-bearing mice injected with PBS or C5aRA, **P*=0.0471(t-test) (**F**). IFN-γ expressing CD8⁺ T cells from lungs of breast tumor-bearing mice injected with PBS and C5aRA, **P*=0.1438, 95% CI -0.01 to 0.002 (t-test) (**G**), and WT and C5aR$^{-/-}$ mice **P*=0.0364 (Mann Whitney test) (**H**). Perforin-expressing (red fluorescence) CD8⁺ (green fluorescence) T cells in lungs of breast tumor-bearing mice injected with PBS (left) or C5aRA (two right panels) (**I**), with quantification **P*=0.0008 (t-test) (**J**). Lung metastatic burden in Balb/c mice treated with C5aRA or PBS and injected with CD8-neutralizing antibody or isotype IgG, **P*=0.0005 (Mann Whitney test), not significant (n.s) (**K**). CD8⁺ T cells in peripheral blood of Balb/c mice treated with PBS or C5aRA and injected with CD8-neutralizing antibody (α-CD8) or isotype IgG (Isotype), **P*=0.0057 (Mann Whitney test), ***P*=0.0040 (t-test with Welch's correction) (**L**). Scale bar 20 μm. Bars represent mean + s.e.m. Data are representative of two independent experiments, n_1=9 and n_2=10 (**A, B, C, F, G**) or three independent experiments n_1=8, n_2=9 and n_3=10 (**E**), or one experiment with at least n=8 (**D,H, I-L**).

(3) C5a regulates the immunosuppressive environment of metastases-targeted organs

The accumulation of immunosuppressive myeloid cells as a contributing mechanism in the formation of premetastatic sites and therefore facilitating metastasis has been reported (5, 6). However, factors regulating the recruitment of these cells to the distant sites require clarification. In our previous study in a model of HPV-induced cancer, we demonstrated that C5a acts as a potent chemoattractant of MDSCs to the primary tumors [15]. Thus, we hypothesized that C5a/C5aR also activates and recruit MDSCs to premetastatic niche that results in immunosuppression in metastases targeted organs prior to tumor cell arrival. In fact, genetic (Fig. 3A) and pharmacological (Fig. 3B, C) ablation of C5aR resulted in decreased MDSC infiltration of the lungs of breast tumor-bearing mice (Fig. 3A, B, C). A similar reduction in MDSCs was observed in the livers of C5aR-/- mice (Fig. 3D, E). In an independent set of experiments, we determined that tumor cells were first observed in the lungs between days 20 to 26 after injection of 4T1 cells into the mammary fat pad (data not shown), whereas a significant increase in MDSC infiltration in the lungs could be detected at day 16 (data not shown). Interestingly, we observed that complement activation, which is associated with C5a generation, occurred in the lungs of breast tumor-bearing mice prior to metastases and significant accumulation of MDSC (Fig. 3F), since complement C3 fragments were deposited in the lungs as early as at day 4 after tumor implantation (Fig. 3F). Therefore, we propose that in premetastatic niche, C5a functions as a chemoattractant for MDSCs expressing high levels of C5aR (Fig. 3G). Since immunosuppressive properties of MDSC in the primary tumor microenvironment are primarily maintained, in a large extent, by cytokines produced in these cells [18], we investigated, if similar mechanisms operate in premetastatic niche. Therefore, we evaluated the impact of C5aR inhibition on the expression of cytokines primarily involved in immunosuppression, such as IL-10, and TGF-β, in the lung myeloid cells of tumor-bearing mice. We determined frequencies of cells that produced only one of the examined cytokines as well as cells that co-expressed both of these cytokines. Total lung cells were isolated from the breast tumor-bearing mice treated with C5aRA or PBS and stimulated ex vivo with a toll like receptor agonist lipopolysaccharide (LPS). We found reduction in the percentage of $CD11b^+$ cells producing only TGF-β in mice treated with C5aRA compared to PBS group (Fig. 3H). Relatively low frequencies of the cytokine producing cells result from the presented data as percentages of functional cells out of total lung cells, since this presentation reflects contribution of these cells to overall lung function. Of note, extremely rare hematopoietic stem cell/progenitors have found to be key contributors to premetastatic niche [7]. Importantly, we found that C5aR inhibition reduced frequencies of $CD11b^+$ cells that co-produced TFG-β and IL-10 (Fig. 3I). TGF-β and IL-10, in addition to facilitating metastasis [19, 20], are also reported to promote Treg cell generation [21, 22], thereby suppressing adaptive immunity in the tumor microenvironment [18]. Therefore, we next assessed whether decreased production of these cytokines in mice treated with C5aRA correlated with the reduced frequencies of Treg cells in the lungs of mice with primary breast tumors. We found that these mice had lower frequencies of Treg cells compared to control mice (Fig. 3J). This finding was consistent with a reduction in the numbers of Treg cells in the circulation (Fig. 3K). Thus, we propose that C5aR signaling contributes to immunosuppression in the metastases-targeted lungs via recruitment of MDSCs to these sites,

regulation of TGF-β and IL-10 expression in these cells and, consequently, generation of Treg cells.

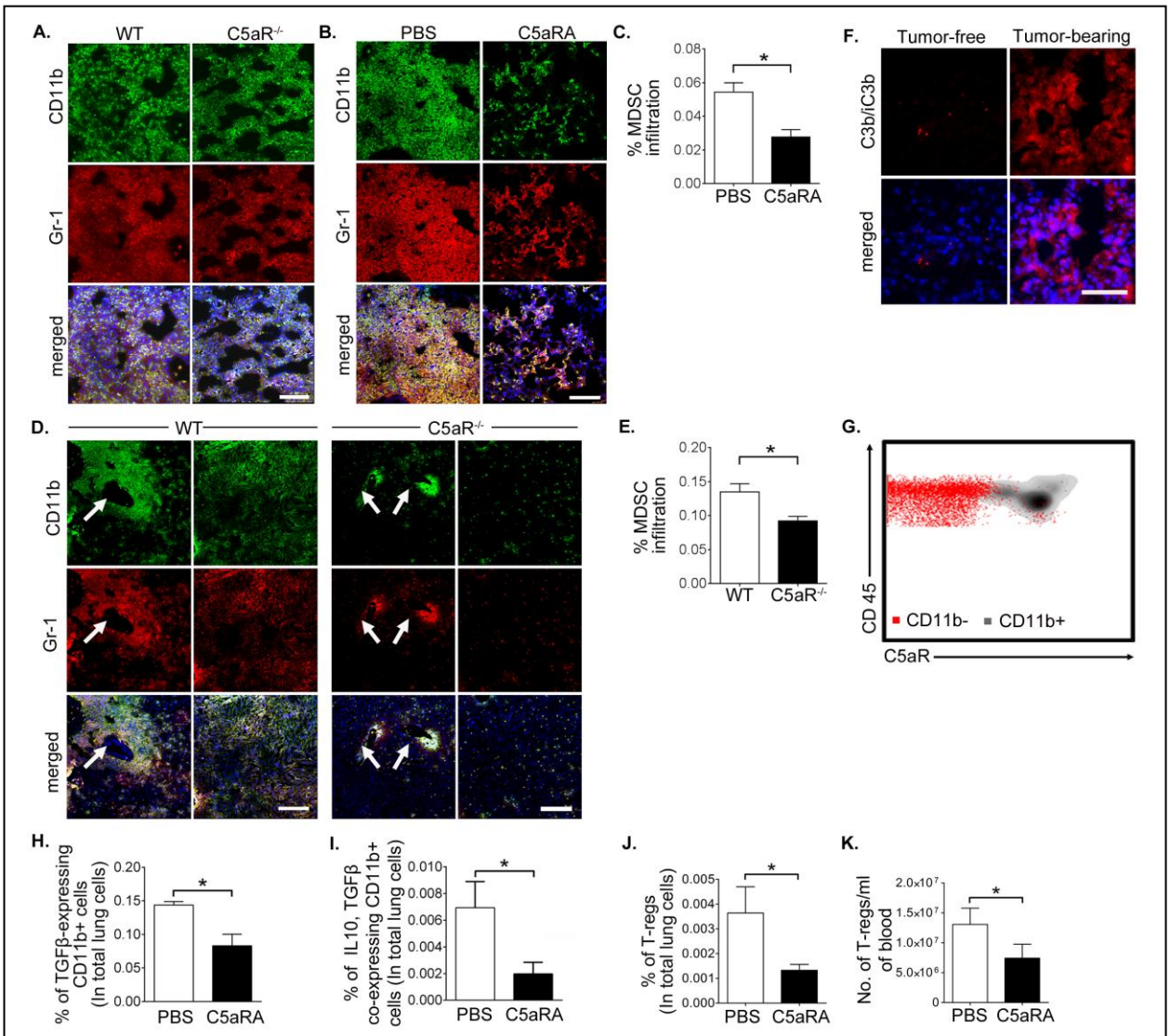

Figure 3. C5aR-mediated immunosuppression in the lungs and livers of breast tumor-bearing mice. CD11b and Gr-1 expression, green and red fluorescence, respectively, in lung MDSCs of tumor-bearing wild type (WT) and C5aR$^{-/-}$ mice (**A**), and tumor-bearing WT mice injected with PBS or C5aR antagonist (C5aRA) (**B**). MDSCs quantification, **P*=0.0017 (t-test) from B (**C**). CD11b and Gr-1 expression, green and red fluorescence, respectively, in liver MDSCs of tumor-bearing WT and C5aR$^{-/-}$ mice, arrows show central veins (**D**). MDSCs quantification based on D, **P*=0.0061 (t-test) (**E**). C3 cleavage products in the lungs of tumor-free and tumor-bearing mice (**F**). C5aR expression in CD11b^{+} blood cells in tumor-bearing mice (**G**). Percentages of CD11b^{+} cells expressing TGF-β after *ex vivo* LPS stimulation, **P*=0.0085 (t-test with Welch's correction) (**H**), and co-expressing TGF-β and IL-10, **P*=0.0412 (t-test with Welch's correction) (**I**) in the lungs of tumor-bearing mice injected with PBS or C5aRA. Percentages of T_{reg} cells in the lungs, **P*=0.2679, 95% CI -0.001 to 0.005 (t-test) (**J**), and absolute counts of T_{reg} cells in the peripheral blood, **P*=0.05 (t-test) (**K**) of tumor-bearing mice injected with PBS or C5aRA. Blue fluorescence-DAPI (**A, B, D, F**). Bars represent mean + s.e.m. Data are representative of one experiment with n=8 (**A, D, E**); or two independent experiments, n_1≥8 and n_2=10 (**B, C, G**); or two independent experiments, n_1=3 and n_2=8 (**F**); or one experiment, n=9 (**H, I**); or one experiment, n≥5 (**J, K**). Scale bar-100μm for A, B, 200μm for D, and 50μm for F.

(4) C5aR in MDSCs affects T cell polarization in metastases-targeted organs

We observed that the genetic C5aR-deficiency led to Th1 polarization of $CD4^+$ T cells in the lungs of breast tumor-bearing mice (Fig. 4A). To confirm that C5a impacts generation and polarization of anti-tumor effector T cell responses in metastases-targeted organs by modulating functions of MDSCs, $CD4^+$ T cells were isolated from the spleens of tumor-free "naïve" mice and differentiated in vitro into effectors by stimulating with CD3/CD28 antibodies in the presence of lung-derived MDSCs ($CD11b^+Gr\text{-}1^+$) obtained from control or C5aRA-treated breast tumor-bearing mice. Importantly, upon FACS analysis, we observed that these T cells ($CD45^+CD11b^-$ population) lack C5aR expression on their surface, which excludes the possibility of a direct action of C5aRA on T cells (Fig. 3G). We found that $CD4^+$ T cells differentiated in the presence of lung MDSCs from C5aRA treated mice displayed increased expression of IFN-γ resulting in a higher Th1/Th2 ratio compared to a similar setting that used lung MDSC from PBS group (Fig. 4B, C). Based on these data we propose that C5aR signaling contributes to the polarization of $CD4^+$ T cells to Th2 type in the lungs of tumor-bearing mice through modulating MDSC functions and disabling C5aR signaling reverses this effect.

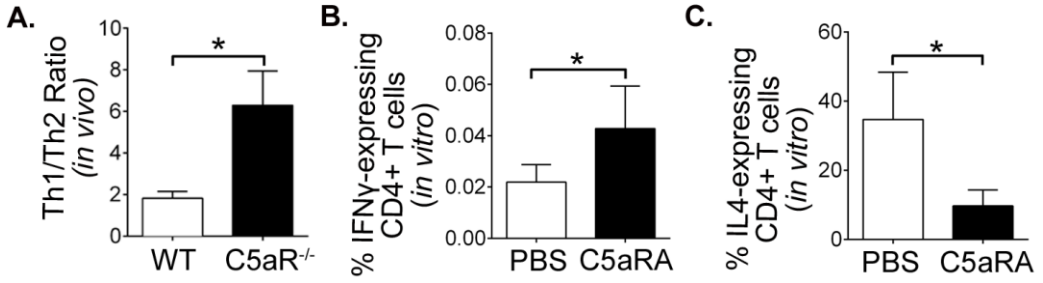

Figure 4. C5aR impacts T cell polarization by regulating lung MDSCs. Th1/Th2 ratio calculated from IFN-γ (Th1)- and IL-4 (Th2)-expressing $CD4^+$ T cells obtained from the lungs of breast tumor-bearing WT and $C5aR^{-/-}$ mice, **P*=0.0245 (t-test with Welch's correction) (**A**). Percentages of IFN-γ, **P*=0.2712, 95% CI -0.02 to 0.06 (t-test) (**B**), and IL-4 expressing, **P*=0.1332, 95% CI -60.23 to 10.18 (t-test with Welch's correction) (**C**) $CD4^+$ T cells differentiated *in vitro* by anti-CD3/CD28 stimulations in the presence of lung MDSCs from breast tumor-bearing mice treated with C5aRA or PBS. Horizontal lines represent mean + s.e.m. Data are representative of one independent experiment with n≥9 (**A**) and *n*≥5 (**B, C**).

(5) Inflammatory changes in the premetastatic niche resemble interstitial pneumonia-like inflammation

In addition to the decreased metastatic burden and MDSCs infiltration in the lungs and livers, C5aR-deficiecy (C5aR-/-) markedly lessen overall inflammatory reactions in these organs. This was demonstrated by reduced inflammatory infiltrates in intra-alveolar septa in the lungs (Fig. 5A) as well as periportal areas of the liver (Fig. 5 B). Morphologic heterogeneity in these infiltrates suggests that apart from MDSCs other cells contribute to premetastatic niche formation and the recruitment of these cells could be C5aR dependent. A detailed histopathological evaluation revealed the presence of progressive inflammatory changes in the intra-alveolar septa of mice bearing tumors. This inflammation acquired an interstitial 'pneumonia-like' pattern in the advanced stage (Fig. 5C). The diffuse interstitial infiltrates in the

lungs were composed of cells resembling granulocytes with an admixture of small lymphocytes and histiocytes. Occasionally, immature myeloid cells were noted (Fig. 5D).

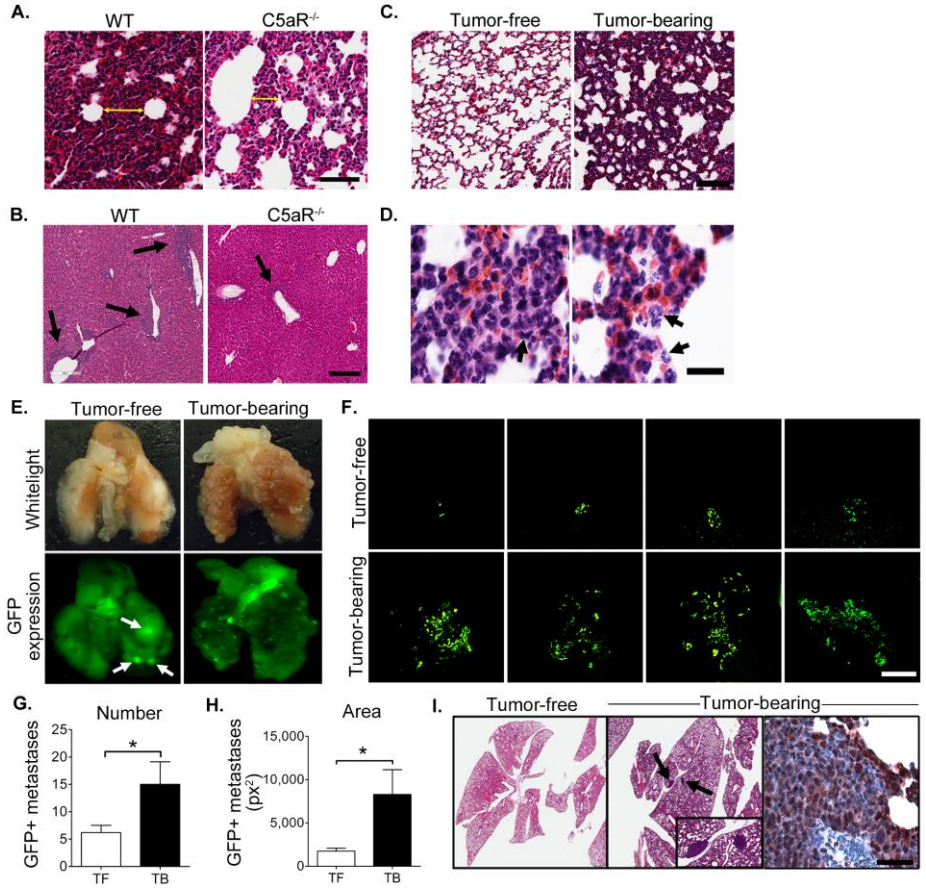

Figure 5. C5aR mediated inflammation facilitates metastases. Hematoxylin and eosin (H&E) stained sections of the lungs of WT and C5aR$^{-/-}$ tumor-bearing mice. Double-headed yellow arrows point to 54.92 μm in WT and 37.61 μm in C5aR$^{-/-}$ mice showing thickness of alveolar septa (**A**). H&E stained sections of the livers of WT and C5aR$^{-/-}$ tumor-bearing mice. Arrows point inflammatory cells (**B**). H&E stained sections of the lungs of tumor-free mice (left panel) and tumor-bearing mice (right panel) (**C**). Immature myeloid cells in H&E stained lung sections of tumor-bearing mice-arrows (**D**). The lungs from tumor-free (left panel) and tumor-bearing mice (right panel) injected i.v. with GFP$^+$ tumor cells. Upper panel - white light images, note multiple gross metastases in breast tumor-bearing mice, lower panel - fluorescence images, arrows point to few GFP$^+$ metastases in tumor-free mice, note numerous GFP$^+$ metastases in breast tumor-bearing mice (**E**). GFP$^+$ tumor cells (green fluorescence) in sections of the lungs of tumor-free (upper panel) and tumor-bearing mice (lower panel) injected i.v. with GFP$^+$ tumor cells (**F**). Numbers of GFP$^+$ metastases in the lungs of tumor-free (TF) and tumor-bearing mice (TB) injected i.v. with GFP$^+$ tumor cells, **P*=0.0604, 95% CI -18.1 to 0.5 (t-test) (**G**). Size (area) of GFP$^+$ metastases in the lungs of TF & TB mice injected i.v. with GFP$^+$ tumor cells, **P*=0.0159 (Mann Whitney test) (**H**). Bars represent mean + s.e.m. Scans of the end-point H&E stained lung sections from TF (left image) and TB mice (middle image) injected i.v. with GFP$^+$ tumor cells. Arrows point to metastases. Inset depicts the enlarged lung area with metastases. Right image- immunohistochemistry detection of GFP in lung metastases (**I**). Data are representative of two independent experiments with n_1=5 and n_2=6 (**A, B,** and **E-I**), or two independent experiments with n_1=3 and n_2=8 (**C, D**). Scale bars-100μm except 50 μm D.

In the next set of experiments, we verified if inflammatory alterations of the lungs observed prior to metastasis in the breast tumor-bearing mice facilitate seeding of these organs by

circulating tumor cells. In these experiments, mice were injected with regular 4T1 cells into the mammary fat pad to create premetastatic niche in the lungs and, next, these mice along with tumor-free control mice were injected i.v. with 4T1-GFP$^+$ (GFP-expressing) cells. This experimental approach enabled to observe, if lung inflammation associated and induced by the primary breast tumor would facilitate lung seeding by circulating GFP$^+$ 4T1 cells (injected i.v.). Colonization of lungs by circulating tumor cells is reported to be dependent on existence of premetastatic niche. We observed that the presence of lung inflammation in tumor-bearing mice increased seeding of 4T1-GFP$^+$ cells in this organ, which was evident from higher number and increased sizes of GFP$^+$ metastases in the lungs of mice previously injected with regular 4T1 into the mammary fat pad (Fig. 5E, F, G, H). Nevertheless, GFP- (non-fluorescent) metastases were also present in these mice (Fig. 5E), however, by using animal imaging combined with fluorescent microscopy; we were able to distinguish GFP$^+$ from GFP- metastases. When these experiments were repeated with a 10-fold lower numbers of 4T1-GFP$^+$ cells injected i.v., only mice bearing breast tumors developed GFP$^+$ metastases in their lungs (Fig. 5I) indicating that circulating tumor cells require prior inflammatory changes in premetastatic niche for avid lung seeding.

Key research accomplishments:

- C5a receptor 1 (C5aR) contributes to metastasis by suppressing T cell responses in the lungs, since reduction in metastatic burden in the lungs by C5aR-inhibition was abolished by CD8$^+$ T cell depletion. C5aR blockade resulted in increased recruitment of CD4$^+$ and CD8$^+$ T cells and induction of Th1/Tc1-biased T cell responses.
- Mechanisms of C5aR-mediated immunosuppression involved recruitment of MDSCs and generation of T_{reg} cells and regulating production of the immunosuppressive cytokines, TGF-β and IL-10, in myeloid cells.

Reportable outcomes:

- *Manuscripts, abstracts, presentations:* "Antitumor activity of a monoclonal antibody targeting major histocompatibility complex class I-Her2 peptide complexes". Jain R, Rawat A, Verma B, Markiewski MM, Weidanz JA. *J Natl Cancer Inst.* 2013 Feb 6;105(3):202-18. doi: 10.1093/jnci/djs521. Epub 2013 Jan 8
- *Funding applied for based on work supported by this award:*
    - NIH grant application entitled "Crosstalk between stem cells and innate immunity in the pre-metastatic niche"; R01 CA181061-01, PI: Maciej Markiewski.
    - NIH grant application entitled "Preventing Cancer Metastases through Inhibition of Complement C5a Receptor"; 1R01CA186889-01, PI: Maciej Markiewski
    - NIH grant application entitled" Targeting premetastatic niche by antiangiogenic immunotherapy therapy"; 1R01CA190209-01, PI: Maciej Markiewski

Conclusions:

We have determined in a syngeneic model of spontaneously metastasizing breast cancer that C5aR1 facilitates metastasis by regulating composition and function of premetastatic niche. To our best knowledge, contributions of C5aR1 to metastasis have not been demonstrated. These studies have identified C5aR1 as a new target for therapies preventing/reducing metastasis.

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